Agilent ChemStation for LC 3D Systems

Understanding Your Spectra Module

Agilent Technologies

Notices

© Agilent Technologies, Inc. 1994, 1995-2003, 2004, 2006,2007

No part of this manual may be reproduced in any form or by any means (including electronic storage and retrieval or translation into a foreign language) without prior agreement and written consent from Agilent Technologies, Inc. as governed by United States and international copyright laws.

Microsoft ® is a U.S. registered trademark of Microsoft Corporation.

Manual Part Number

G2180-90023

Edition

02/07

Printed in Germany

Agilent Technologies
Hewlett-Packard-Strasse 8
76337 Waldbronn, Germany

Software Revision

This guide is valid for B.03.xx revisions of the Agilent ChemStation for LC 3D Systems software, where xx refers to minor revisions of the software that do not affect the technical accuracy of this guide.

Warranty

The material contained in this document is provided "as is," and is subject to being changed, without notice, in future editions. Further, to the maximum extent permitted by applicable law, Agilent disclaims all warranties, either express or implied, with regard to this manual and any information contained herein, including but not limited to the implied warranties of merchantability and fitness for a particular purpose. Agilent shall not be liable for errors or for incidental or consequential damages in connection with the furnishing, use, or performance of this document or of any information contained herein. Should Agilent and the user have a separate written agreement with warranty terms covering the material in this document that conflict with these terms, the warranty terms in the separate agreement shall control.

Technology Licenses

The hardware and/or software described in this document are furnished under a license and may be used or copied only in accordance with the terms of such license.

Restricted Rights Legend

If software is for use in the performance of a U.S. Government prime contract or subcontract, Software is delivered and licensed as "Commercial computer software" as defined in DFAR 252.227-7014 (June 1995), or as a "commercial item" as defined in FAR 2.101(a) or as "Restricted computer software" as defined in FAR 52.227-19 (June 1987) or any equivalent agency regulation or contract clause. Use, duplication or disclosure of Software is subject to Agilent Technologies' standard commercial license terms, and non-DOD Departments and Agencies of the U.S. Government will receive no greater than Restricted Rights as defined in FAR 52.227-19(c)(1-2) (June 1987). U.S. Government users will receive no greater than Limited Rights as defined in FAR 52.227-14 (June 1987) or DFAR 252.227-7015 (b)(2) (November 1995), as applicable in any technical data.

Safety Notices

CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

In This Guide...

This handbook describes the concepts behind the spectra module of the Agilent ChemStation revision B.03.0x. It complements the information in the *Understanding Your ChemStation* manual with the special spectra concepts applicable to the Agilent ChemStation for LC 3D, CE systems, and the UV-visible part of the Agilent ChemStation for MSD.

For details of installation and configuration of your spectra module with an Agilent ChemStation for LC 2D system, see the *Installing Your ChemStation* manual.

1 Understanding Spectral Analysis

This chapter covers the following:

- What is spectral analysis?
- Determining the optimum detection wavelength.
- Spectral report styles.

2 Spectral Libraries

Traditional data systems quantify the detected compounds by correlations based on retention times. This can lead to erroneous identification, if:

- compounds other than the calibrated ones appear in the specified retention time window,
- more than one peak appears in the specified retention time window, or
- the compound is retained on the column longer than the specified window because of solvent flow rate changes or, more commonly, changing column characteristics.

One way to avoid such errors is to use peak qualifiers as described in the *Understanding your Agilent ChemStation* manual.

With a diode array detector such errors can be avoided, since UV-visible spectra can be used to confirm a peak's identity. Standard spectra are acquired from a reference sample under well-defined chromatographic conditions and stored in a database (spectral library). You can compare peak spectra of an unknown sample with those stored in one or more libraries. Spectra can be overlaid for visual comparison and the similarity of standard and sample spectra can be calculated.

The ChemStation can automate this process for all peaks within a chromatogram and for all chromatograms within a sequence. In addition to such peak confirmation, the software can perform a peak purity check, using the settings in the Purity Preferences dialog box.

These qualitative results can be combined with quantitative results into a single report. This report contains all the information an analyst needs for each peak from a run such as:

- compound name,
- amount,
- retention time,
- identity match factor, and
- purity match factor.

This chapter describes the concepts of a spectra library search. For information on how to perform library searches refer to the online help system and the integrated tutorial.

3 Evaluating Peak Purity

This chapter describes peak-purity evaluation for B.03.0x revisions of the software.

Contents

1 Understanding Spectral Analysis 7

What is Spectral Analysis? 8

Determining the Optimum Detection Wavelength 10

Spectral Report Styles 12

2 Spectral Libraries 13

Search Modes 14

Report Description 17

- Purity Marker 17
- Library Match Marker 17
- Compound Name Marker 18
- Amount Marker 18

3 Evaluating Peak Purity 19

Checking Peak Purity 20

- What is a Peak Purity Check? 20
- The Match Factor 21
- Background Correction by Selecting a Reference Spectrum 22
- Peak-Purity Techniques 24

Peak Purity Display 25

- Spectra Window 25
- Spectral Normalization 26
- Purity Window 27
- Spectral Similarity Curves 28
- Threshold Curve 29
- Using Specific Target Spectra 31

Purity Calculation and Display 32
Classifying a Peak as Pure or Impure 33
Peak Purity Information 34
Advanced Peak Purity Options 36
Using Peak Purity Analysis 38
Spectra Acquisition 38
Setting Options for Peak Purity 39
Mass Spectral Peak Purity 41
Mass Spectral Purity Calculations 42
Mass Spectral Purity Display 43
Index 45

1 Understanding Spectral Analysis

What is Spectral Analysis? 8
Determining the Optimum Detection Wavelength 10
Spectral Report Styles 12

What is Spectral Analysis?

Spectral analysis allows you to process spectral data acquired from the UV-visible diode-array detector or the fluorescence detector.

The analysis of spectral data adds a third dimension to your analytical data when using it with chromatographic data (see Figure 1).

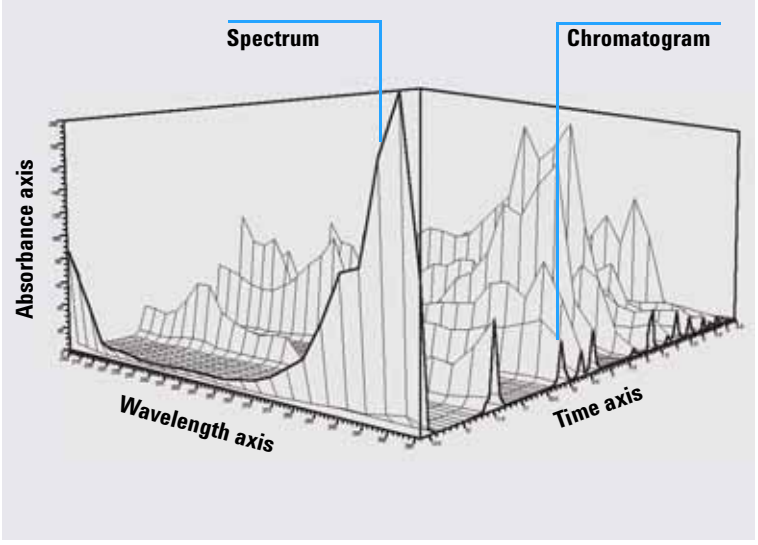

Figure 1 Spectral Information

UV-Visible Diode-Array Detector

The UV-visible diode-array detector allows you to continuously acquire spectra in the UV-visible region of the spectrum; because the diode-array detector acquires all wavelengths simultaneously, there is no loss of sensitivity during spectral acquisition.

Fluorescence Detector

The fluorescence detector allows you to collect spectral data in three different modes:

- you can set a fixed excitation wavelength and collect emission spectra
- you can set a fixed emission wavelength and collect excitation spectra
- you can acquire a fluorescence scan, in which both excitation wavelength and emission wavelength are varied to give a three-dimensional characterization of the sample.

Excitation and emission spectra can be acquired during chromatography, but because the fluorescence detector is a scanning detector, the more wavelengths that are acquired (the wider the scan range) the lower the sensitivity of the analysis. The three-dimensional fluorescence scan must be acquired either in stopped flow mode or off-line, since it requires a constant concentration of sample in the cell throughout the analysis.

Spectral Processing

You can process your spectral data in many ways. For example, you can:

- extract chromatographic signals from spectral data to determine the optimum detection wavelength for each peak,
- do spectral library searches to obtain a qualitative identification,
- calculate ratios of chromatographic signals to determine peak purity, and
- do peak-purity checks to discover (hidden) impurities.

Determining the Optimum Detection Wavelength

After you have developed suitable conditions for peak separation, the next step in method development is to determine the optimum detection wavelength for each peak.

One technique is to present the peak intensity (absorbance or luminescence), wavelength and time as a contour map called an isoabsorbance plot. This technique plots the spectral information as a series of isoabsorptive, concentric lines in the wavelength and time plane. This allows for all spectral information to be presented and inspected simultaneously. See the center display in Figure 2.

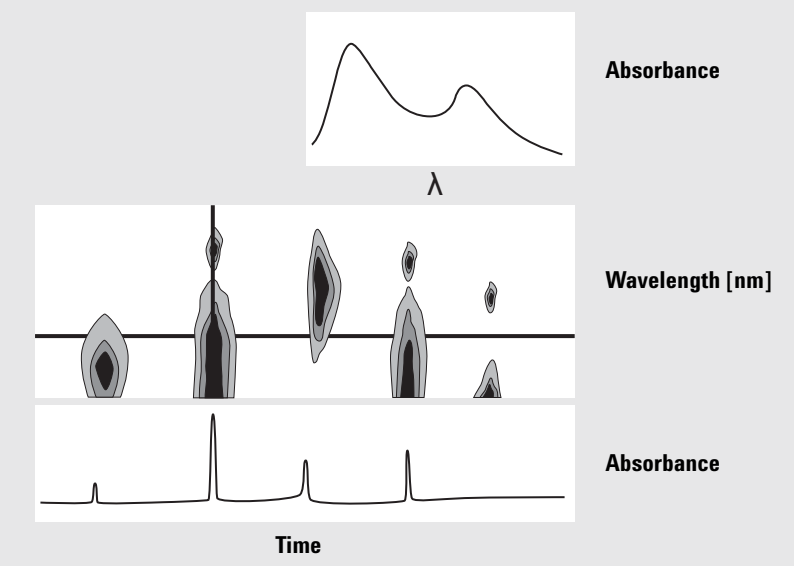

Figure 2 Isoabsorbance Plot

This technique is useful in method development to find the optimum detection wavelength for each separated peak. The wavelength corresponds to the horizontal cursor position in the isoabsorbance plot. When moving the cursor along the wavelength axis, the chromatogram is reconstructed in the lower window, see Figure 2 on page 10.

To find the optimum detection wavelength for a peak it is necessary to do the following:

- Select Quick View from the Cursor field and move the horizontal cursor in the center display until the absorbance of the peak of interest is as high as possible, and there is a good chromatographic separation in the lower display.
- Select Signal from the from the Cursor field and optimize the bandwidth to increase the signal-to-noise ratio to obtain the optimum wavelength for detection. The selected signal and bandwidth may be transferred to the signal window in the data analysis view by clicking the Copy button. The signal and bandwidth can then be used to test the integration, identification, and quantification processes.

Wavelength optimization with the fluorescence detector generally requires two runs:

- Set up an excitation wavelength in the low UV range (230 to 250 nm) and collect emission spectra. For each peak in the chromatogram, determine the optimum emission wavelength using the isoabsorbance plot.
- Prepare a timetable to set the optimum emission wavelength for each peak as it elutes, and collect the excitation spectra.

Fluorescence Spectrum

The three-dimensional iso plot is used to display spectra obtained from fluorescence scans only. In this case, the spectral information is plotted on the excitation wavelength/emission wavelength plane. There is no time axis, since the fluorescence scan is acquired either in stopped-flow mode or off line. From the iso plot, you can extract excitation or emission spectra at any wavelength; the excitation spectra are shown above the iso plot (vertical cursor), the emission spectra are shown below the iso plot (horizontal cursor).

Spectral Report Styles

The following report styles are available with the spectral module:

- Library Search

  produces a calibrated report including library search results. Peak numbers, retention times, library search match factors, amounts, and compound names are printed according to the parameters specified in the Automated Library Search dialog box. For more information see “Report Description” on page 17.

- Short + Spectrum

  comprises instrument conditions, chromatogram, quantitative results, and peak purity information.

- Detail + Spectrum

  comprises header, instrument conditions, chromatogram, quantitative results, calibration curves, and peak purity information. The header is stored in a file called RPTHEAD.TXT in the method directory. You can change the header using a text editor to include a method specific text.

- Performance + Lib. Search

  combines the Performance and Library Search styles.

Peak Purity Information

Peak purity information refers to the evaluation of spectra across the peaks resulting in purity plots for each compound. These plots comprise overlaid and normalized spectra, and single and overlaid signals. The purity plots can also include the threshold and similarity curves, depending on the Purity Preferences settings for the method.

The numeric calculations include the purity factor, a measure of the similarity in the spectra shape. For more information on peak purity refer to Chapter 3, “Evaluating Peak Purity”.

2 Spectral Libraries

Search Modes 14
Report Description 17
Purity Marker 17
Library Match Marker 17
Compound Name Marker 18
Amount Marker 18

Search Modes

In a spectral library search one of three search modes may be used. A spectral library *and* a calibration table are prerequisites for all three modes. You can add a peak purity check as an option.

- Identify by spectral library search.

  This mode is the most general search mode. The ChemStation uses the chromatogram as basis for the library search. It compares the peak spectra of *all peaks found by the integrator* and characterized by their retention times, with spectra of up to four specified libraries. To speed up the process or to increase reliability, you can narrow down the search to spectra that fall within a certain retention time window using the Library Search Template see Figure 3.

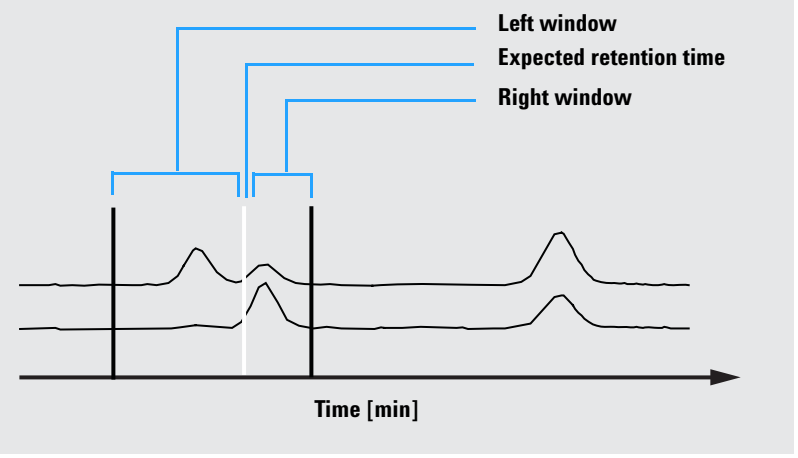

Figure 3 Retention Time Window

The name of the compound with the best match is assigned to the peak. You can specify how good this match must be with your own threshold value. If a match factor is found, which is better than the threshold, the peak is

reported as being *identified*. The ChemStation then refers to the calibration table for an identically named entry. If such an entry is found, the data is used to calculate and report the amount.

If no matching name is found in the calibration table, the peak's identity is given, however, since the method is not calibrated for the compound, an amount is not calculated.

This mode is recommended when searching for unknown compounds in an analysis. It is most efficient if the number of compounds in the chromatogram is low compared to the number of entries in the library.

- Target compound analysis using a calibration table.

  This search mode is only for compounds for which the method is calibrated. The ChemStations compares the peaks spectra *only for calibrated compounds* with spectra of up to four specified libraries. Peaks are only reported as identified if confirmed by spectral comparison. The compound names defined in the calibration table must be identical to the compound names defined in the library.

  A target window for retention times can be specified as part of the search method. Only peaks with retention times that fit into the target window are used in the identification process. The best match is reported as being identified only when the match exceeds the threshold that you have set.

  This mode is recommended for searching specific calibrated compounds in the chromatogram. It is preferable to the first search mode if the chromatogram contains many compounds but only a few, the ones of interest, are calibrated.

- Target compound analysis using a spectral library.

  This search mode is for all library entries, not only those that are also contained in the calibration table. The ChemStation uses the spectra library as basis. It compares *all library spectra* with peak spectra from the chromatogram and attempts to identify all peaks found by the integrator which fall into the retention time window specified for the library entries and in the library search template. Only the peaks for which the retention time fits into the target window are used in the identification process. When a match is found that exceeds the threshold you have set, the peak is reported as identified. Quantification proceeds based on the name of that compound in the calibration table. If the compound exists both in the

library and in the calibration table, it is reported as calibrated and identified. If the compound is not included in the calibration table, it is reported as uncalibrated.

This mode is recommended when you have set up libraries containing specific groups of compounds. For example, you may have one library containing water-soluble vitamins and another library containing fat-soluble vitamins. You can then search the chromatogram for these particular compounds. If the chromatogram contains many compounds and the library contains only a few compounds, this mode is preferable to the first search mode.

- Peak Purity Check

  The peak purity check is optional. We recommend using the peak purity check before a library search, since a match is only as reliable as the quality of the separation. The ChemStation compares the consistency of the spectra in different sections of each peak. It calculates and reports a purity factor. Purity can be defined by a threshold, below which questionable results are tagged with an x in the report. For more information on peak purity refer to Chapter 3, “Evaluating Peak Purity”.

Report Description

A typical report generated by a spectral library search includes:

- chromatogram/electropherogram annotated with retention/migration times,
- header with information on file names and search parameters,
- description of the detector's signal and two analysis tables,
- retention times of the peaks found in the analysis,
- retention times of library match candidates,
- retention times recorded in the calibration table, and
- compound amounts and their purity factor (if selected), together with:

  library entry number,
  library match factor, and
  compound name.

In different columns of the library search report, certain markers are used to denote compound purity, compound matching, and compound naming.

These markers can be interpreted as described in the next steps.

Purity Marker

For impure compounds the spectra at the upslope and downslope of the peak are normally different and therefore both are used for the library search.

u: Compound impure and spectrum at the upslope was used for identification.

d: Compound impure and spectrum at downslope was used for identification.

Library Match Marker

x: Match factor below the given match threshold.

Compound Name Marker

?: Compound identified with match factor below the given match threshold.

??: Compound identified with a name already allocated to another compound with a better match. For this specific compound, a better match with a unique name could not be found.

Amount Marker

+: Individual amount high — amount limit set in Compound Details is exceeded.

-: Individual amount low — amount limit set in Compound Details is exceeded.

Peaks that do not match any entries in the calibration table are listed in a separate table labeled Uncalibrated Compounds.

3 Evaluating Peak Purity

Checking Peak Purity 20
What is a Peak Purity Check? 20
The Match Factor 21
Background Correction by Selecting a Reference Spectrum 22
Peak-Purity Techniques 24
Peak Purity Display 25
Spectra Window 25
Spectral Normalization 26
Purity Window 27
Spectral Similarity Curves 28
Threshold Curve 29
Using Specific Target Spectra 31
Purity Calculation and Display 32
Classifying a Peak as Pure or Impure 33
Peak Purity Information 34
Advanced Peak Purity Options 36
Using Peak Purity Analysis 38
Spectra Acquisition 38
Setting Options for Peak Purity 39
Mass Spectral Peak Purity 41
Mass Spectral Purity Calculations 42
Mass Spectral Purity Display 43

This section describes the various methods that you can use to evaluate the purity of a peak.

Checking Peak Purity

An important question in any chromatographic analysis is whether the peak comprises one or more components. In quality control, impurities hidden behind the peak of interest can falsify results. In research analysis, a hidden and undetected component might lead to a loss of essential information of value to research work.

What is a Peak Purity Check?

A peak purity check assesses whether a peak is pure or contains impurities. This assessment is based on the comparison of spectra recorded during the elution of the peak. Five spectra per peak are used to assess purity: two spectra on each of the up and down slopes and one at the top (top, or apex spectrum). The five spectra are averaged and compared with all spectra recorded in the peak.

If the peak spectra are not identical with the average spectrum, the peak theoretically contains a spectral impurity. The spectral impurity can be caused by one or more components, non-baseline separated peaks, or by background absorption.

NOTE If the spectra are identical, the peak can still contain impurities. This can be the case if the spectral absorption is small compared to the main compound, or if the impurity and the main compound have the same or almost the same spectra, with similar elution times.

The Spectra window contains the peak spectra that comprise the comparison (average) spectra drawn in a normalized and overlaid mode. The Purity window contains the signals with superimposed purity information. The purity factor is a measure of the similarity in the shape of the spectra.

Peak impurity can be detected even if there is a background absorption in the system, which can be corrected. Usually the background absorption does not disturb the quantification of the peak, since it also influences the height of the

peak start and end which is removed by baseline correction. The background absorptions can change by using different solvents or solvent composition in one analysis.

The Match Factor

Peak impurity detection by a visual spectral comparison is time-consuming and is not suitable for automated operations. Several statistical techniques are available for automated comparison of spectra. One technique is a mathematical comparison between two spectra. It calculates a match factor representing the degree of similarity between the spectra.

The comparison of two spectra gives the match factor, which is defined as:

$$\text{Match Factor} = \frac{10^3 \times \left\{\sum x \times y - \left(\frac{\sum x \times \sum y}{n}\right)\right\}^2}{\left\{\sum x^2 - \left(\frac{\sum x \times \sum x}{n}\right)\right\} \times \left\{\sum y^2 - \left(\frac{\sum y \times \sum y}{n}\right)\right\}}$$

The values x and y are measured absorbances in the first and second spectrum respectively, at the same wavelength; n is the number of data points and Σ the sum of the data. At the extremes, a match factor of 0 indicates no match and 1000 indicates identical spectra. Generally, values above 990 indicate that the spectra are similar. Values between 900 and 990 indicate there is some similarity, but the result should be interpreted with care. All values below 900 indicate the spectra are different.

The match factor is influenced by a number of parameters, which are determined by the sample and the separation method. They include compound specificity, spectral absorption of matrix compounds, and spectral noise level, as well as background absorption and spectral shifts caused by solvent or different instruments (different wavelength calibrations).

Smoothing Spectra

The reliability of a purity check is limited when the spectral noise is of the same order as the spectrum. Smoothing spectra works in the following way.

1 For a defined number of data points, for example 5, also referred to as the filter, a cubic regression is used to determine a new data point.
2 The filter then moves one point further, taking the last 4 of the already used data points and an additional point and the process is repeated.

By using the smoothing algorithm, statistical noise can be reduced, which makes the identification of the smoothed spectrum more reliable.

NOTE The smoothing algorithm can also change the profile of a spectrum, depending on the length of the filter, i.e. the number of data points used in the smoothing algorithm at one time. It is recommended that you smooth all spectra with the same filter before making comparisons.

Splining Spectra

If you have acquired spectra with low resolution, you can use splining to make your spectrum look more like a curved line rather than a polygon. This is done by calculating additional data points between the original data points of your spectrum using trigonometric functions. When splining spectra, the original data points are preserved.

Logarithmic Spectra

Logarithmic spectra compress the absorbance scale. They may be useful where the absorbances cover a very wide range.

Derivative Spectra

Derivative spectra reveal more specific details than original spectra when comparing different compounds. Small differences in the spectra are much more obvious and easier to identify visually. However, the noise is increased, which limits the use of derivative spectra.

Background Correction by Selecting a Reference Spectrum

Various types of correction are available to extract peak spectra from the data file for background corrections:

Manual Reference Selection

When one reference spectrum is selected, the selected spectrum at the specified time is taken from the data file and subtracted from each peak spectrum. This correction cannot be used for a changing background absorption.

When two baseline spectra are selected, a linear interpolation is made between the two spectra. A reconstructed reference spectrum based on the linear interpolation is subtracted from each peak spectrum. This correction can be used to compensate for a slow changing background absorption.

Automatic Reference Selection

In automatic reference selection, the selected reference spectra depend on the storage mode of the spectra in the data file.

All Spectra: The integrated peak start and end spectra of the selected peak are taken as the reference spectra, and linearly interpolated as for manual reference selection with two reference spectra.

Peak-controlled Spectra: The nearest baseline spectrum is taken as the reference spectrum and subtracted as for manual reference selection with one reference spectrum.

Peak-Purity Techniques

Peak purity techniques can only be used with peaks that have a baseline separation. If peaks are not baseline-separated, then the peak purity is more complicated because each peak contains the neighboring peaks as an impurity.

Peak purity may be determined interactively on a peak-by-peak basis for all the peaks from a certain data file, or automatically at the end of each analysis as part of the method when using a spectral report style, for example detail+spectrum.

You can optimize peak purity processing for accuracy or performance by setting options relating to:

- wavelength range used for the purity determination,
- reference spectra,
- purity threshold,
- spectral processing, including logarithmic, smoothing and splining factors, and derivative order,
- purity components, calculated and displayed (these include the spectra, spectral differences, the signals, similarity and threshold curves).

The techniques that you can use to judge a peak's purity, which are described in the following sections, include:

- spectral normalization

  comparison of normalized spectra from different peak sections,

- similarity curve

  comparison of an average or selected spectrum with all other spectra taken as the peak eluted.

Peak Purity Display

Spectra Window

The spectra window shows the five spectra that are used to calculate the average spectrum that is used in the purity calculation. You can use the Agilent ChemStation's graphical manipulations to examine the peak spectra in more detail.

Spectral Normalization

A common peak purity technique involves normalizing and comparing spectra taken across the peak. Normalization compensates for the changing concentration of the component passing through the detector cell as the peak eluted. This function is available as part of the Agilent ChemStation's spectral display facilities.

Spectra are acquired, for example, at the upslope, the apex, and at the downslope of the peak. The spectra are normalized and overlaid for graphical presentation. This technique is very good for interactive data evaluation and can also be adapted for automated routine analysis.

Alternatively, if a numeric factor is preferred, upslope and downslope spectra can be mathematically compared and purity factors can be printed for each peak together with retention time.

Figure 4 shows an example in which spectra have been acquired at the upslope, the apex and the downslope of the peaks, and normalized. Both the visual comparison and the purity factor calculated by the software clearly show the difference between the pure and impure peaks.

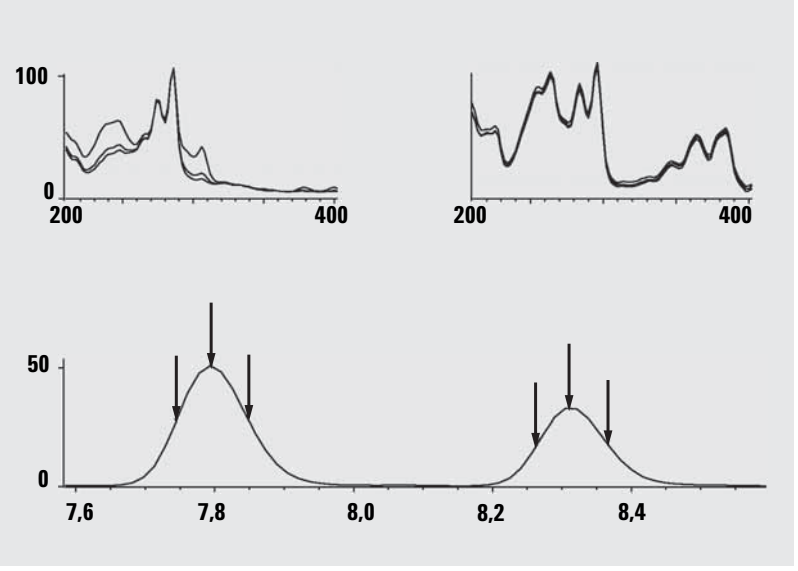

Figure 4 Peak-Purity Check by Overlaying Spectra

Purity Window

The contents of the Purity window depend on the purity parameters selected. The default display is the Purity Ratio, which shows the signals with superimposed similarity and threshold curves (see Figure 5). The green and red bands below the display show the user-defined purity threshold. The calculated purity value for each spectrum in the peak is shown as a black dot; if the black dot lies within the green band, the purity of the spectrum lies within your acceptable purity definition.

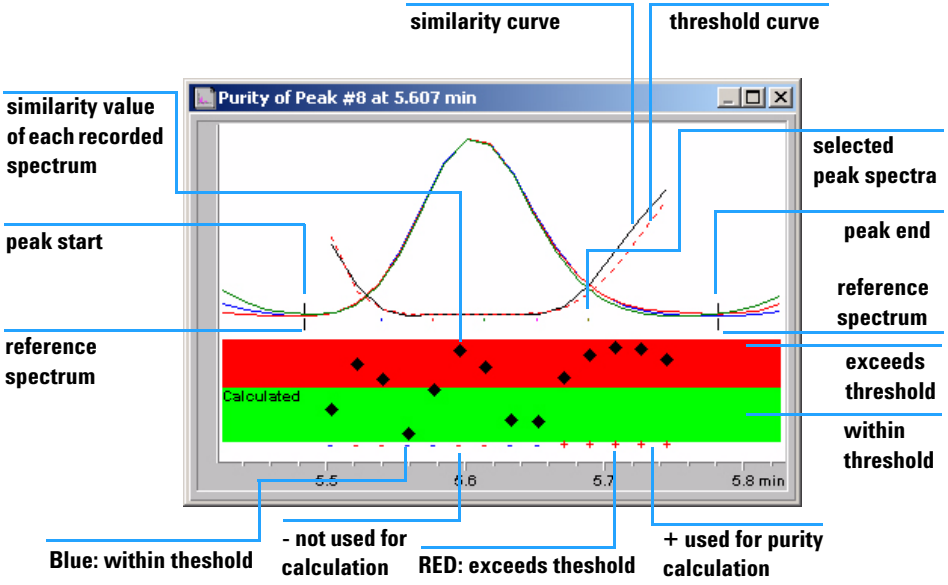

Figure 5 Purity Window

Spectral Similarity Curves

Spectral similarity curves are shown in the Purity window of the Spectra display (see Figure on page 41).

If details are not clear in the display, you can zoom in by holding down the left button while moving the mouse. An area is drawn on the screen as you move the mouse to show the zoom area. To revert to the original display, double click the left mouse button.

Spectral Similarity Curves give the most detailed information about a peak's purity or impurity. All spectra from a peak are compared with one or more spectra, by default an average spectrum. The degree of match or *spectral similarity* is plotted over time during elution. An ideal profile for a pure peak is a flat line at 1000, as shown in Figure 6 (a).

At the beginning and end of each peak, where the signal-to-noise ratio decreases, the contribution of spectral background noise to the peak's spectra becomes significant. The contribution of noise to the similarity curve is shown in Figure 6 (b).

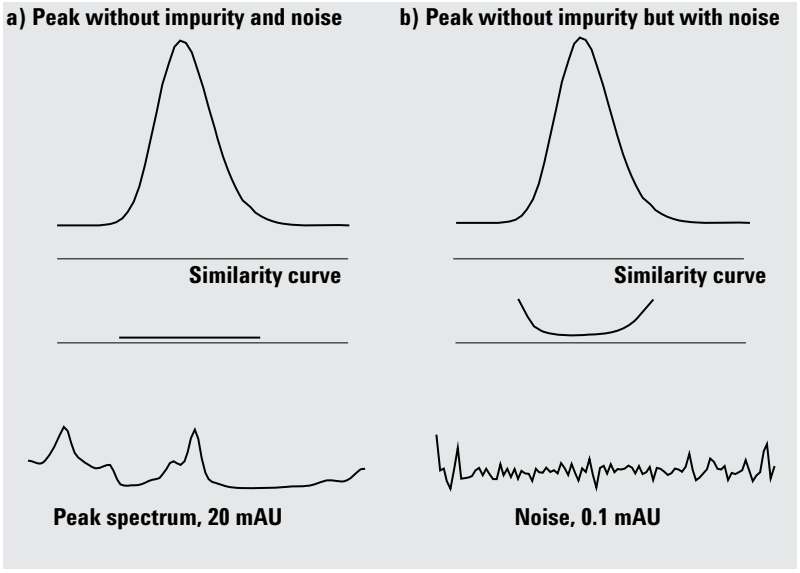
a) Peak without impurity and noise
b) Peak without impurity but with noise
Similarity curve
Similarity curve
Peak spectrum, 20 mAU
Noise, 0.1 mAU

Figure 6 Similarity Curves for a Pure Peak With and Without Noise Plotted in Relation to Ideal Similarity Factor (1000) and User-Defined Threshold (980)

Threshold Curve

The threshold curve shows the effect of noise on a given similarity curve. The effect increases rapidly toward the start and end of a peak. In essence, a threshold curve is a similarity curve of a pure peak with a background noise component.

The influence of noise can be seen in Figure 7. The similarity factor decreases with a decreasing signal-to-noise ratio or constant noise level with decreasing absorbance range.

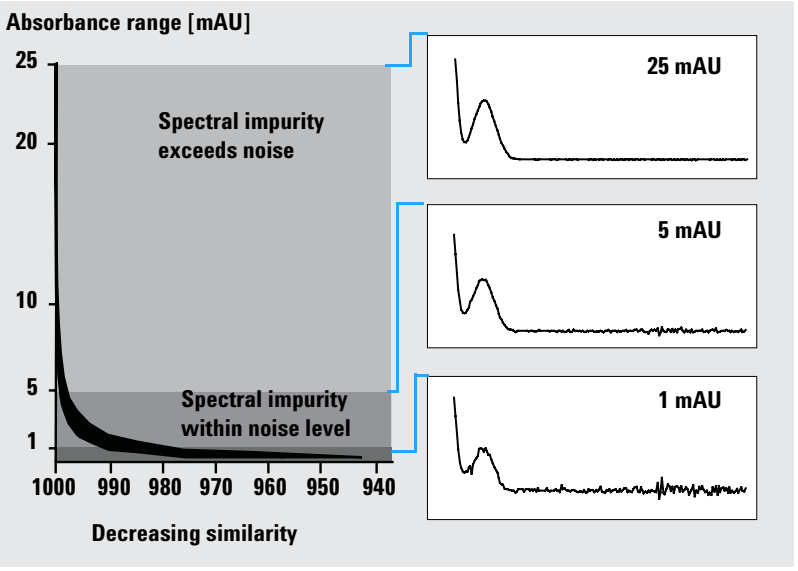

Figure 7 Similarity Factor as a Function of the Noise Level

Figure 8 (a) shows both the similarity curve and the threshold curve for a pure peak with noise, Figure 8 (b) for an impure peak.

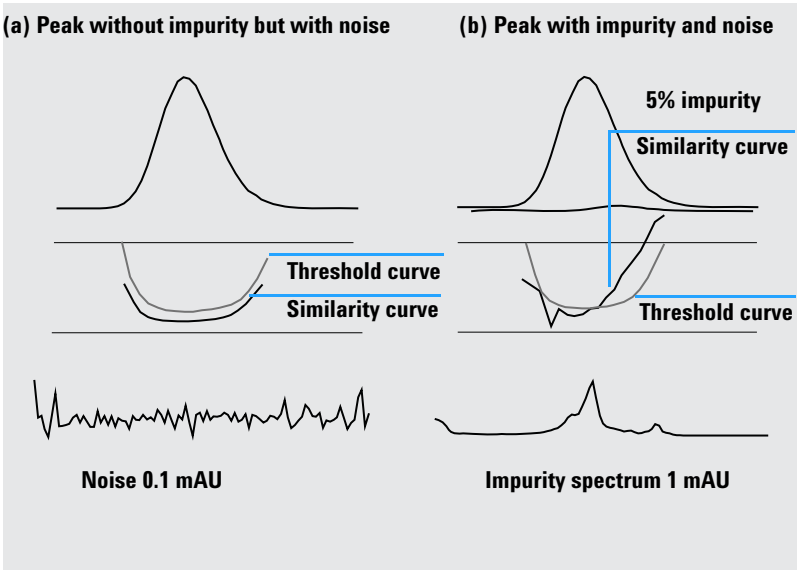

Figure 8 Effect of Impurity and Noise on Similarity and Threshold Curves

The noise threshold is determined automatically, based on the standard deviation of pure noise spectra at a specified time, by default 14 spectra at the beginning of the run (i.e. at 0 minutes).

The threshold curve, represented by the broken line, gives the range for which spectral impurity lies within the noise limit. Above this threshold, spectral impurity exceeds the spectral background noise and the similarity curve intersects the threshold curve, indicating an impurity (provided the reference and noise parameters have been sensibly chosen).

Using Specific Target Spectra

The Agilent ChemStation permits calculations of the purity factor and similarity curves relative to different target spectra, as shown in Figure 9. As a general rule, the default comparison with the average spectrum provides the most valuable information for most unknown impurities. The flexibility of being able to select a specific target spectrum is valuable in cases where the analyst must assume where the impurity is, or needs to improve the sensitivity of the purity evaluation. An example may help to show how this principle can be applied: if the impurity is assumed to be in the tail of the peak, selecting the tail or apex spectrum to be compared with all other spectra will provide the most significant information in this case.

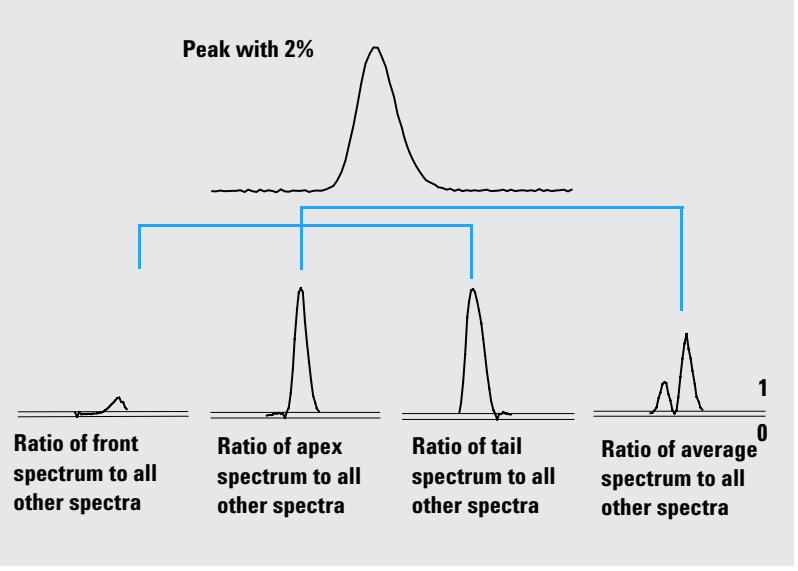
Peak with 2%
1
0
Ratio of front spectrum to all other spectra
Ratio of apex spectrum to all other spectra
Ratio of tail spectrum to all other spectra
Ratio of average spectrum to all other spectra

Figure 9 The Ratio Curves for Different Target Spectra from the Same Peak

Figure 9 gives the ratio curve for the front, apex, tail, and average spectrum of a peak which contains an impurity after the response maximum (apex).

The **front spectrum** gives a small spectral impurity at the end of the peak. The deviation in this first ratio curve is small since the front spectrum absorbed little (giving a rather high threshold curve).

The **apex spectrum** gives a low impurity in the front of the peak (the apex spectrum contains only a very small amount of the impurity) and high impurity at the tail.

The **tail spectrum** (with a high level of impurity) gives a spectral impurity at the front of the peak.

The **average spectrum** (a mean of the five selected peak spectra) indicates spectral impurity in the total peak. This average spectrum contains the spectral contribution of the impurity. In this case, the average contains more contribution from the impurity than the apex spectrum, showing a higher spectral impurity at the elution or migration front, and lower impurity at the tail, compared with the ratio curve of the apex spectrum.

The profile of the similarity, threshold and ratio curves depends on the position, level, and spectral differences of the impurity and, as such, no general statements can be made on the shape. Expect the profile to differ from situation to situation.

Purity Calculation and Display

The purity factor is not an absolute measure of the peak purity. It is a function of the parameters used in the calculations, especially the purity threshold. Therefore, the results need to be interpreted together with the threshold set. For pure peaks, comparable results are generated for different parameter settings. For impure peaks, a worst case analysis is done. Only data points that refer to an impurity, for example spectra that lie below the threshold, are considered in the calculation, therefore a change in the threshold value can drastically influence the purity factor for an impure peak. See below for more details on these calculations. For a detailed description of the Agilent ChemStation similarity curves, see “Spectral Similarity Curves” on page 28.

All Spectra

1. All reference-corrected spectra recorded for the peak that are above the user-defined threshold are used for the calculation of the purity and threshold curves; five of these spectra are displayed in the Spectra window.
2. The match factors are calculated between each spectrum and the average of the five spectra displayed in the Spectra window; they are plotted as single points in the Purity display.

For the User-defined threshold, the similarity curve is superimposed on the Purity plot.

For the Calculated threshold, the threshold value is calculated for each spectrum; the purity values of the spectra are defined as a ratio between the threshold value and the similarity value which give a straight line. This gives a better indication of the pure and impure data points. The similarity and threshold curves are superimposed on the purity plot.

Peak-Controlled Spectra

1 All reference-corrected spectra recorded for the peak that are above the user-defined threshold are used in the calculation and displayed in the Spectra window.

2 The match factors are calculated between each spectrum displayed in the Spectra window and the average of these spectra; they are plotted as single points in the Purity display.

3 The purity value is calculated as the mean value of all the calculated match factors.

Classifying a Peak as Pure or Impure

The calculated purity level is used to generate the purity statement displayed on the screen. If the purity factor is within the threshold value, (set manually, or calculated from the threshold curve), the peak is classed as pure. If the purity factor exceeds the threshold value, the peak is classed as impure.

NOTE The detected impurity is a *spectral* impurity which does not necessarily mean a compound impurity. Spectral impurities can be caused by changes in solvent composition (gradients) or can occur in peaks that are not baseline separated.

Peak Purity Information

Purity Factor

The purity factor gives a numerical value for the purity of the peak.

- If the peak has been classified as pure, the purity factor is the mean value of all spectra that are *within* the threshold.
- If the peak has been classified as impure, the purity factor is the mean value of all spectra that *exceed* the threshold.

In either case, the number of spectra used for the calculation of the purity factor, and the basis for the calculation are specified.

Threshold

For a calculated threshold, this is the mean value of all those spectra used in the calculation of the purity factor. For a user-defined threshold, this is the value you set in the Purity Options.

Peak Spectra

Peak Spectra shows details of the five spectra selected for the purity calculations. You can also display

- Difference spectra

  Spectra calculated as the difference between the averaged spectrum and the individual selected spectra. Pure peaks show only noise in the difference spectra.
- Compared spectrum

  The average spectrum used for the purity calculations.
- All recorded spectra

  All spectra recorded across the peak.
- Reference spectra

  The spectrum or spectra used for background correction.

Purity and Noise Calculations

The information about the Purity Calculation shows the number of recorded spectra and the number of those within the threshold or exceeding the threshold that have been used to calculate the purity factor.

The information about the Noise Calculation shows the spectra that have been used to calculate the noise threshold, and the results of the calculation. You can show the noise spectra as well as a graph of the noise statistics. You can also change the parameters for the noise calculation.

NOTE Changing the parameters for the calculation of background noise can have a major effect on the results of the purity calculation. Ensure that you understand the information given in "Advanced Peak Purity Options" on page 36 before making any changes.

Purity Curve

The Purity Curve information shows the results of the comparison of each spectrum in the peak with the average spectrum. The values in the Difference column represent the arithmetical difference between the values in the Purity column and those in the Threshold column. The Difference value is used to determine which spectra are used in the calculation of the Purity factor and Threshold value for the peak.

More Purity Curves

The information for More Purity Curves shows the results of purity calculations using Front, Apex and Tail spectra in addition to that of the average spectrum (see "Using Specific Target Spectra" on page 31). You can choose to show the similarity curves of any or all of these calculations. You can also change the parameters of the purity calculation, and the way that the results are displayed.

NOTE Changing the parameters for the calculation of purity can have a major effect on the purity results. Ensure that you understand the information given in "Advanced Peak Purity Options" on page 36 before making any changes.

Advanced Peak Purity Options

Changes made in the Advanced Peak Purity Options can have a major effect on the purity results. We recommend that you do not make changes unless you understand the effect that your changes will have on the results.

Purity Calculation

The purity calculation by default uses the average of five spectra across the peak (see "What is a Peak Purity Check?" on page 20), but you can also use other spectra as the basis of the calculation:

All peak spectra uses each of the five selected spectra to produce five sets of results that are displayed in the same window.

Apex spectrum, Front spectrum and **Tail spectrum** use specific spectra that may allow you to improve the sensitivity of the purity analysis (see "Using Specific Target Spectra" on page 31).

Front & Tail spectrum shows the two sets of results produced using the Front and Tail spectra as the basis of the purity calculation.

The default display of peak purity is the Purity Ratio, as shown in Figure 5 on page 27, but you can also display the purity results as similarity and threshold curves.

The Agilent ChemStation provides three modes to display the similarity and threshold curves:

1. Without any transformation, see Figure 10 on page 37 (a);
2. As the natural logarithm, *ln*, see Figure 10 on page 37 (b), with the advantage of more detail for the peak apex in the lower part of the graphic;
3. As a ratio: $\text{ratio} = \frac{1000 - \text{similarity}}{1000 - \text{threshold}}$, see Figure 10 on page 37 (c).

For a spectrally pure peak, the ratio values are below 1, and for spectrally impure peaks, the values are above 1. The advantage of the ratio mode is that only one line is displayed, leading to easier interpretation.

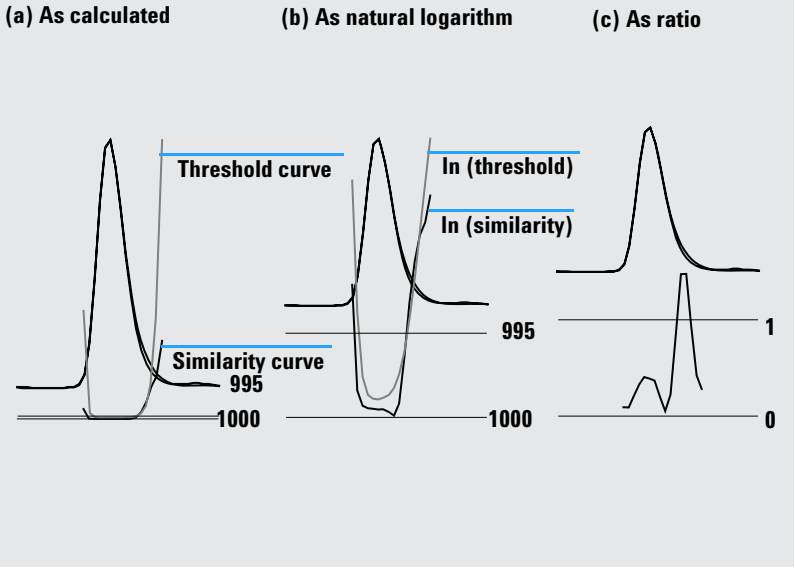

Figure 10 Threshold and Similarity Curves,
(a) as Calculated,
(b) l_n (Threshold) and l_n (Similarity), and
(c) as a Ratio

Noise Threshold

By default, the noise threshold is determined automatically, using the standard deviation of 14 pure noise spectra at the beginning of a run (0 minutes). You can change the time and number of the spectra from which the standard deviation of noise will be calculated, or you can enter a fixed value for the standard deviation (default value 0.1). Best accuracy is achieved when the standard deviation is calculated from a specified number of scans.

Using Peak Purity Analysis

This section includes practical hints on acquiring data suitable for peak purity analysis and setting the peak purity options.

Spectra Acquisition

Peak purity testing is based on comparison of spectra across a peak. Make sure that enough spectra are available for comparison and that the quality of the spectra is high. Make sure the detector is well maintained, the lamp intensity is strong, and that you choose the appropriate flow cell and slit. In general, you should optimize for sensitivity rather than resolution, since even small shifts in the spectra are expressed over broader wavelength ranges, and resolution is generally not so important.

Use the store All spectra to collect spectra continuously. Newer detectors support the All in Peak mode which collects spectra continuously when a peak is detected and in addition stores about 20 spectra at the start of the run for use in determining baseline noise for the purity analysis.

Set the peak width on the diode array detector screen to the width of the narrowest peak of interest in the run.

Make sure your sample concentration is appropriate for the linear operation range of the detector. If a component is too concentrated, the detector will operate outside its linear range at the wavelengths of highest absorbance while remaining linear at other wavelengths. This will cause the shape of the spectra to change with concentration, and the component will be reported as possibly impure. If the component concentration is too low, the signal-to- noise ratios for the spectra will be poor and the sensitivity of the purity analysis will be reduced. The most accurate and sensitive purity analysis is for peaks between about 250 and 800 mAU in height.

Use the default settings for checking peak purity. If a possible impurity is detected, you can take a more critical look at the results to confirm the impurity and characterize it further.

Setting Options for Peak Purity

Select the following settings in the appropriate tabs of the Spectral Options dialog box.

Wavelength Range

Use this setting to control the portion of the wavelength range used for the purity analysis. You can set a low limit for example to exclude wavelengths where absorbance of the mobile phase causes excessive noise. You can set a high limit to exclude higher wavelengths where the compound of interest does not absorb.

Spectra Processing

Use spectral processing to perform mathematical calculations to transform, smooth or spline spectra. In practice, anything that enhances small differences in the spectra (derivatives) also enhances the noise, while anything that reduces the noise (smoothing) decreases the sensitivity for small spectral changes.

Absorbance Threshold

The absorbance threshold sets lowest intensity of spectrum that can be included in the analysis. Normally, the threshold is set to 1 – 2 mAU to ensure that the spectra at the edges of the peak are included in the purity check.

Reference Spectrum

Reference spectra are baseline spectra used to correct the background absorbance. We recommend that you always use a reference spectrum. The recommended setting is Automatic. With this mode, a spectrum is taken from the baseline near the start and end of integration. A linear extrapolation of these two spectra is used to correct each spectra across the peak. As this is a two-point reference, it can compensate for detector drift and for changes in mobile phase composition using gradients. The other reference modes are available for backwards compatibility with previous software and so that some purity information can be obtained even if only a few spectra were collected.

If two peaks are not completely baseline-separated, automatic selection of reference spectra using Automatic reference selection might lead to a reference spectrum being selected from the valley between the two peaks. An

unresolved peak cannot be pure. In this case the purity test can be used to look for other hidden components. Use Manual reference selection to select reference spectra from before and after the group of peaks.

Purity Threshold

If your data was collected by acquiring peak-controlled spectra, you need to enter a value for the purity threshold. Generally, the default value of 990 will give acceptable results.

If your data was collected by acquiring All spectra or All in peak, the best results will be produced by allowing the Agilent ChemStation to calculate a threshold for each spectrum based on its individual signal-to-noise ratio. Alternatively, you can set a fixed threshold for all scans in the peak, although this is not recommended. If you do wish to use a fixed threshold, use a much higher value than for peak-controlled spectra, e.g. between 995 and 998.

Mass Spectral Peak Purity

In LC/MS, the abundances of the ion(s) that characterize a compound maximize at a particular retention time, when the concentration of that component in the MSD is at a maximum. Depending on the chromatography conditions and the scan cycle time, the retention times of closely-eluting peaks may be very close together, and multiple components may appear as a single peak or as a peak with distortions (e.g. shoulders) in the total ion chromatogram (TIC). By examining the retention times at which individual ions maximize, it may be possible to group together clusters of ions that maximize at the same retention time; these ions are all then assumed to belong to the same component. If more than one cluster of ions maximize at different retention times under the same chromatographic peak envelope, the peak can be said to be impure, and the individual clusters of ions can be determined.

In this analysis, several assumptions are made:

- The components of a multicomponent mixture can be separated either by their mass spectra or their retention times. Either each component of the mixture has a distinctive mass spectrum with unique *m/z* values, or the components are sufficiently well separated to allow multiple maxima to be determined.
- The signal-to-noise ratio is high enough to allow real maxima to be identified unambiguously.
- The ion retention times that are used as the basis for calculating the component retention times are precise and representative.

With only a rare exception, electrospray ionization (ESI) produces only molecular ions unless ion source collision-induced dissociation (CID) is used to produce fragments. This means that if two distinct, singly charged ions are found in a series of spectra (even if they co-elute) it is possible to identify and quantitate those ions. Care should be taken when doing this since cation and anion adducts can generate ions besides the expected $[M+H]^+$ or $[M-H]^-$ ions. Atmospheric pressure chemical ionization (APCI) also produces molecular ions but thermal fragments are more common in APCI than electrospray.

In addition, a mass spectrum contains isotope information that can be used to help with identification and purity assessment.

The peak purity algorithm will only identify an impure peak if there are spectral and time differences. Manual interpretation can go beyond that limit. Unlike UV data, MS data can be used to quantitate impure peaks as long as the “compounds” have different ions. Peak Purity generally requires scan data, not SIM data, to be useful.

Mass Spectral Purity Calculations

A range of the TIC covering the integrated area of the peak is searched using a selected set of *m/z* values, and the maximum abundance for each *m/z* value is determined. For each maximum found, an interpolated retention time (scan number) is calculated using a parabolic fit. The retention times of the largest maxima are grouped into clusters, which are then identified with the components that are present in the range. Information about how many components, the retention time (scan number) of each component and the largest *m/z* values in the cluster are reported.

The selected set of *m/z* values is obtained by examining the scans at 25%, 50% and 75% of the integrated peak time range, using all *m/z* values that have an abundance greater than 1% of the maximum abundance in the scan (base peak). For each selected *m/z* value, the average abundance of the first and last scans in the time range is used for background correction before the clusters are determined.

Each *m/z* value in the set is examined in every scan in the time range, and the maximum abundance is stored along with the abundances on either side of the maximum. Only one maximum per *m/z* value is stored. Each stored maximum is verified by checking that it does not occur in the first or last scan, and does not have zero abundance on either side.

After an interpolated scan number has been calculated for the *m/z* value and the background correction has been made, a table is produced containing *m/z* values, abundance and interpolated scan numbers, ordered by interpolated scan number.

Clusters are determined by examining the largest abundances in the list in order of increasing scan number. Once a cluster has been registered, the average interpolated scan number of the *m/z* values in the cluster is calculated; if the next point under consideration is more than half a scan away from the average, a new cluster is started.

The number of clusters found is reported as the number of components; the average interpolated scan number is the reported scan number; and the *m/z* values with the two largest abundances in the cluster are reported as the ions.

An integrated peak that contains more than one cluster is reported as impure.

Mass Spectral Purity Display

Ions for Peak Window

The Ions for Peak window shows the two *m/z* values with the largest abundances in each cluster. Single component peaks show two ions maximizing at the same retention time; multi-component peaks show pairs of ions maximizing at the same retention time. Each pair of ions indicates a component in the impure peak. You can use the Agilent ChemStation's graphical manipulations to examine the ions in more detail.

MS Peak Purity Results Window

The MS Peak Purity Results window contains the tabular results of the peak purity analysis. Each line in the table contains the information for one component: average interpolated scan number and the two most abundant ions.

Index

A

apex spectrum, 32
automated spectral library search
  overview, 3
  peak purity, 16
  search modes, 14
  standard search, 14
  target compound analysis, 15
average spectrum, 32

B

background
  absorptions, 20
background correction, 22
baseline
  correction, 23

C

compare
  spectra, 26
COMPARE command, 21
compound name marker, 18
contour
  lines, 10
  map, 10

D

derivative spectra, 22
determining
  optimum wavelength, 10

F

fluorescence spectrum, 11
front spectrum, 31

H

hints, 38

I

impurity, 20
isoabsorbance plot, 10

L

library match marker, 17
linear range, 38
logarithmic spectra, 22

M

mass spectral peak purity, 41
match factor, 21

N

normalize
  spectra, 26

O

optimum wavelength, 10

P

peak
  impurity, 20
  purity, 20
peak purity
  mass spectral, 41
  spectral similarity curves, 28
  threshold curve, 29, 36
  using specific target spectra, 31
  what is?, 20
peak signals window, 28
purity
  peak, 20
purity calculations
  mass spectral, 42
  threshold curve, 29, 33
purity display
  mass spectral, 43

R

reference spectrum, 39
  background correction, 22
report
  library search, 12
  library serach, 12
  peak purity, 12
  spectral, 12

S

smooth spectra, 21
spectra
  derivative, 22
  logarithmic, 22
  normalize, 26
  smooth, 21
spectra acquisition, 38
spectral analysis
  what is?, 8
spectral operations
  compare, 26
  normalize, 26
spectral similarity curves, 28
spectrum
  correction, 23
  reference, 39
spectrum, fluorescence, 11

T

tail spectrum, 32
target spectra, 31
third dimension, 8
threshold curve, 29, 33, 36
    display mode, 30

W

wavelength
    optimization, 10

www.agilent.com

In This Book

This handbook describes the concepts behind the spectra module of the Agilent ChemStation revision B.03.0x. It complements the information in the *Understanding Your ChemStation* manual with the special spectra concepts applicable to the Agilent ChemStation for LC 3D and CE systems and the UV-visible part of the Agilent ChemStation for MSD.

For details of installation and configuration of your spectra module with an Agilent ChemStation for LC 2D system, see the *Installing Your ChemStation* manual.

© Agilent Technologies 1994, 1995-2003, 2004, 2006,2007

Printed in Germany
02/07

G2180-90023

Agilent Technologies